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Transcription from the Gene Encoding the Herpesvirus Entry Receptor Nectin-1 (HveC) in Nervous Tissue of Adult Mouse

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Both human and murine forms of nectin-1 (HveC, Prr1) can serve as entry receptors for several neurotropic herpesviruses, including herpes simplex viruses 1 and 2 (HSV-1, HSV-2), porcine pseudorabies virus (PRV), and bovine herpesvirus 1. HSV-1, HSV-2, and PRV can cause lethal neurological disease in mice whether inoculation is directly into the central nervous system or by peripheral routes. Expression of nectin-1 transcripts in cells of the adult mouse nervous system was assessed by *in situ* hybridization. Specific hybridization signals were detected in neurons in sensory, sympathetic, and parasympathetic ganglia of the peripheral nervous system. In addition, specific signals were observed in neurons of the ventral and dorsal horns of the spinal cord and of the brain stem, cerebellum, cerebral cortex, hippocampus, dentate gyrus, and olfactory bulb. These results show that the nectin-1 gene is widely transcribed in neurons in adult mouse. Nectin-1 is the only known receptor capable of mediating the entry of all three viruses, HSV-1, HSV-2, and PRV. Its pattern of expression in the nervous system suggests a key role in neurological disease caused by these viruses.

Key Words: HSV; herpes simplex virus; pseudorabies virus; nectin-1; viral entry; mouse; neurons; *in situ* hybridization.

INTRODUCTION

Herpesviruses are divided into the subfamilies α-, β-, and γ-herpesvirinae. The alphaherpesviruses, such as herpes simplex viruses 1 and 2 (HSV-1, HSV-2), are neurotropic. In their natural hosts and in experimental animals, these viruses can replicate in epithelial cells at the portal of entry to cause mucocutaneous lesions and also enter neurons at peripheral sites and migrate by retrograde transport to sensory and autonomic ganglia, where latent infections are established. Under certain conditions, these viruses may spread to the central nervous system and cause lethal encephalitis. The spread of viral infection in the nervous system can be via synapses. Both HSV and another alphaherpesvirus, porcine pseudorabies virus (PRV), have been used in experimental animals to trace synaptic connections (reviewed by Enquist *et al.,* 1998). Mice, which are susceptible to neurological disease caused by HSV-1, HSV-2, and PRV, have been the experimental animal of choice for many studies.

Entry of alphaherpesviruses into cells usually requires multiple interactions between viral envelope proteins and cell surface receptors. HSV-1, HSV-2, and PRV bind to cells through interactions of specific viral glycoproteins (gC and/or gB) with cell surface heparan sulfate (Herold *et al.,* 1994; Mettenleiter *et al.,* 1990; Shieh *et al.,* 1992; WuDunn and Spear, 1989). Then another viral glycoprotein, gD, can bind to any one of several specific cell surface receptors as described below. This binding leads to fusion of the viral envelope with a cell membrane, which requires the action of the viral glycoproteins gB, gH, and gL in addition to gD and its receptor (Cai *et al*., 1988; Forrester *et al.,* 1992; Ligas and Johnson, 1988; Roop *et al*., 1993).

Recently, three classes of cell surface molecules, both human and mouse forms, have been shown to serve as gD receptors for entry of one or another of the alphaherpesviruses mentioned above. Table 1 lists these molecules and indicates which can serve as entry mediators for each of the three viruses. Herpesvirus entry mediator (HVEM), also called HveA, is a member of the tumor necrosis factor (TNF) receptor family (TNFRSF14). Both mouse and human forms of HVEM can mediate the entry of HSV-1 and HSV-2 but not PRV (Montgomery *et al.,* 1996) (D. Shukla and P. G. Spear, unpublished data). Nectin-1 and nectin-2 are related members of the immunoglobulin superfamily (Eberlé *et al.,* 1995; Lopez *et al.,* 1995; Takahashi *et al.,* 1999). Both mouse and human forms of nectin-1 can serve as entry receptors for all three viruses (Cocchi *et al.,* 1998; Geraghty *et al.,* 1998; Menotti *et al.,* 2000; Shukla *et al.,* 2000). In contrast,

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TABLE 1

Human and Mouse Entry Receptors for HSV-1, HSV-2 and PRV

Name	Other names	Protein family	Mediates entry of: HSV-1	HSV-2	PRV
HVEM	HveA	TNF receptor family	Yes[a]	Yes	No
Nectin-1	HveC; Prr1	Ig super-family	Yes	Yes	Yes
Nectin-2	HveB; Prr2	Ig super-family	No	Yes (H) No (M)[b]	Yes
3-O-sulfated HS			Yes	No	No

[a] Unless indicated otherwise, the virus can use both the human and the mouse forms of the receptor for entry (Yes) or can use neither (No).

[b] Human nectin-2 is a weak receptor for HSV-2 and for certain mutant strains of HSV-1 but not for HSV-1 wild-type strains. The mouse form of nectin-2 has no entry activity for any of the HSV-1 or HSV-2 strains tested.

nectin-2 has more limited entry activity. Although both mouse and human forms of nectin-2 can serve as entry receptors for PRV, human nectin-2 is only a weak entry receptor for HSV-2 and certain strains of HSV-1 and mouse nectin-2 exhibit no entry activity for HSV-1 or HSV-2 (Lopez *et al.,* 2000; Shukla *et al.,* 1999b; Warner *et al.,* 1998). A specific modification in heparan sulfate generated by certain 3-*O*-sulfotransferases can also bind to gD to serve as an entry receptor, but only for HSV-1 strains, not for HSV-2 or PRV (Shukla *et al.,* 1999a).

Analyses of RNA extracted from various organs or cultured cells have provided some information about the patterns of expression of these entry receptors in mouse and human tissues. HVEM RNA has been detected in a variety of organs, including spleen, thymus, intestine, lung, and kidney, but is not readily detected in brain or cell lines of neuronal origin (Geraghty *et al.,* 1998; Hsu *et al.,* 1997; Kwon *et al.,* 1997; Marsters *et al.,* 1997; Montgomery *et al.,* 1996). Nectin-2 is widely expressed in many organs, including brain, and nectin-2-specific RNA has been detected in a variety of cell lines including some of neuronal origin (Eberlé *et al.,* 1995; Morrison and Racaniello, 1992; Satoh-Horikawa *et al.,* 2000; Shukla *et al.,* 1999b; Warner *et al.,* 1998).

Expression of nectin-1 appears to be somewhat more restricted but is especially prominent in brain, kidney, liver, pancreas, and skin and in cell lines of neuronal or epithelial origin (Geraghty *et al.,* 1998; Lopez *et al.,* 1995; Menotti *et al.,* 2000; Satoh-Horikawa *et al.,* 2000; Shukla *et al.,* 2000). As is true for other members of the nectin family, there are at least two membrane-bound isoforms of human nectin-1, designated nectin-1α and nectin-1β (Satoh-Horikawa *et al.,* 2000), and one secreted form designated nectin-1γ (Lopez *et al.,* 2001). The three forms have identical ectodomains composed of one V-like Ig domain and two C-like Ig domains. They differ only at the C-terminal ends, having different membrane-spanning regions and cytoplasmic tails or a short hydrophilic domain that permits secretion. All isoforms can serve as alphaherpesvirus entry receptors, even the secreted form which tends to bind to the cell surface (Cocchi *et al.,* 1998; Geraghty *et al.,* 1998; Lopez *et al.,* 2001). To date, only one form of nectin-1, homologous to human nectin-1α, has been described in mice (Menotti *et al.,* 2000; Shukla *et al.,* 2000). Comparisons of nectin-1α amino acid sequences among various mammalian species (human, monkey, mouse, cow, pig, and hamster) have revealed a high degree of conservation with greater than 90% identity in the ectodomain for all pairwise comparisons (Milne *et al.,* 2001). Both nectin-1 and nectin-2 (and a third related protein called nectin-3) are cell adhesion molecules that localize to sites of cadherin-based adherens junctions in epithelial cells and perhaps other junctions in other cell types (Miyahara *et al.,* 2000; Nishioka *et al.,* 2000; Satoh-Horikawa *et al.,* 2000; Tachibana *et al.,* 2000; Takahashi *et al.,* 1999).

The specificities of these herpesvirus entry receptors for different viruses and the levels of their expression in various organs suggest that nectin-1 is the prime candidate for a receptor that could allow entry of HSV-1, HSV-2, and PRV into neurons and perhaps other cells of the nervous system, leading to the severe neurological disease that can be observed in mice infected with any of these viruses or, under certain conditions, in their natural hosts. The particular cell types that express nectin-1 or the other entry receptors in various organs or tissues are largely unknown. The *in situ* hybridization study described here was undertaken to identify cells in the mouse nervous system that express nectin-1-specific RNA. The results showed that neurons throughout the peripheral and central nervous system, as well as some nonneuronal cells, transcribe the gene for nectin-1. Thus, nectin-1 could be the principal receptor for the entry of these viruses into neurons of the peripheral and central nervous systems of mice and probably also of their natural hosts.

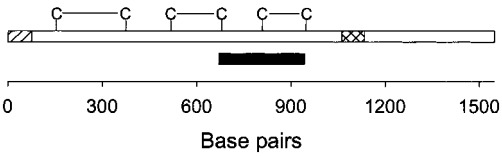

FIG. 1. The nectin-1 probe used for *in situ* hybridization. The upper bar shows the nectin-1 open reading frame and cysteine residues of the protein in relation to base pairs of the cDNA sequence (lower line). Predicted domains for signal sequence (diagonal hatch) and membrane-spanning (cross-hatched) are indicated. A 276-bp fragment of the ectodomain of murine nectin-1 cDNA was amplified by PCR and inserted into a vector for *in vitro* transcription as described under Materials and Methods. The location of the probes generated (nucleotides 668–943 counting from the first nucleotide of the start codon) is indicated by the black bar. Digoxigenin-labeled antisense and sense probes were synthesized as described under Materials and Methods.

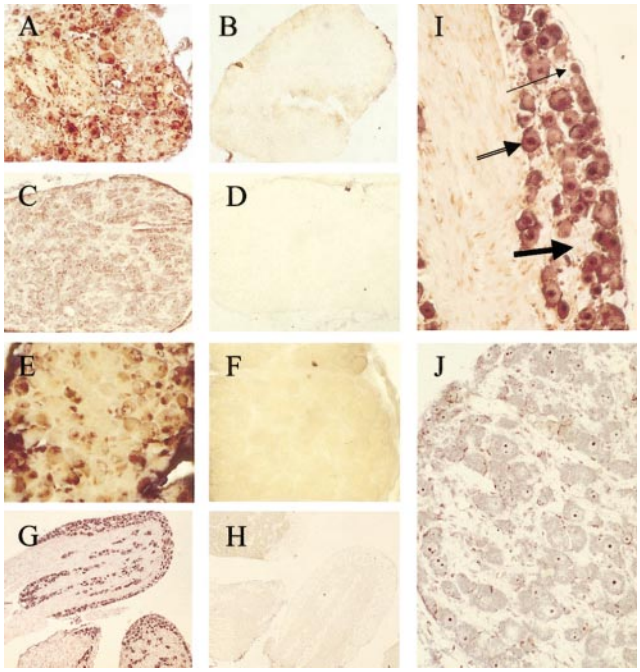

FIG. 2. *In situ* hybridization of sections from different ganglia. Sections from different ganglia were subjected to *in situ* hybridization using either the antisense (A, C, E, G, I, and J) or the sense (B, D, F, and H) probe. (A and B) Spinal ganglion, magnification ×80. (C and D) Superior cervical ganglion, magnification ×80. (E and F) Pterygopalatine ganglion, magnification ×320. (G and H) Trigeminal ganglia, magnification ×80. (I) Trigeminal ganglion, magnification ×320. The thin, medium, and thick arrows indicate predominantly nuclear staining, both nuclear and cytoplasmic staining, and weak staining, respectively. (J) Superior cervical ganglion, magnification ×320.

RESULTS

Design of the nectin-1 probe for *in situ* hybridization

We prepared a template for synthesis of hybridization probes by subcloning a portion of the cDNA (276 bp) encoding part of the nectin-1 ectodomain (nucleotides 668–943 counting from the first base of the start codon), as shown in Fig. 1. The antisense probe prepared from this template is homologous to nectin-1α mRNA and would also be homologous to other mRNAs that might be generated from mouse nectin-1 primary transcripts by differential splicing downstream of the region depicted in Fig. 1. The template has less than 50% homology to other members of the nectin family. Probes for *in situ* hybridization were labeled with digoxigenin (DIG) and detected after hybridization by use of an anti-DIG antibody. Both the antisense and the sense probes were incubated with tissue sections from the mouse nervous system. Positive signals were observed with the antisense probe only, provided the tissue sections had not been pretreated with RNase. Positive signals were not observed with the sense probe whether or not the tissue sections had been pretreated with RNase.

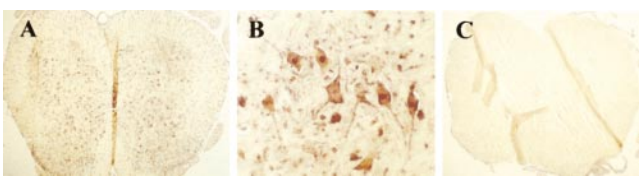

FIG. 3. *In situ* hybridization in the spinal cord. Cross sections from the thoracic spinal cord were hybridized with the antisense (A and B) or the sense (C) probe. Magnifications are ×55 (A and C) and ×200 (B).

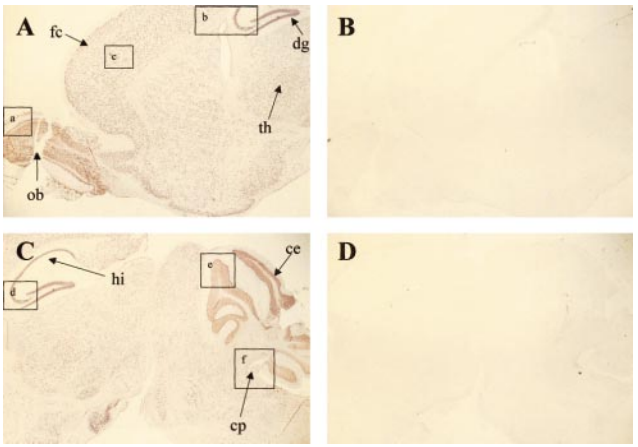

FIG. 4. *In situ* hybridization in the central nervous system as observed at low magnification (×20). Sagittal sections included the brain, cerebellum, and brain stem. The forebrain is shown in A and B, and the hindbrain including cerebellum and the brain stem are shown in C and D. The antisense probe was used in A and C, and the sense probe was used in B and D. Some main structures are indicated (fc, frontal cortex; dg, dentate gyrus; th, thalamus; ob, olfactory bulb; hi, hippocampus; ce, cerebellum; cp, choroid plexus). The boxed areas labeled a–f are shown at higher magnifications in Fig. 5.

Nectin-1 RNA in neurons in various ganglia

Sections were prepared from sensory, sympathetic, and parasympathetic ganglia and *in situ* hybridization performed using the antisense or sense probe from the nectin-1 gene. The results are shown in Fig. 2 for a spinal ganglion (A and B), a superior cervical ganglion (C and D), a pterygopalatine ganglion (E and F), and trigeminal ganglia (G and H). Distinct neurons and nerve fibers were clearly observed in all preparations incubated with the antisense probe even in the absence of counterstaining with hematoxylin–eosin. Hybridization signals were obtained with the antisense probe in the majority of neurons in all ganglia, although some cells were weakly or undetectably stained (A and E). Lightly stained cells in the fibrous parts of the ganglia (A and G) were most likely Schwann cells. No signal was obtained with the sense probe (B, D, F, and H). Higher magnification images from the trigeminal and the superior cervical ganglia are shown in Figs. 2I and 2J, respectively. Nectin-1 mRNA was present in the cytoplasm of most of the reacting cells. Additional nuclear localization was observed in many cells, particularly in the sensory trigeminal (Fig. 2I)

and spinal (Fig. 2A) ganglia. Examples of weak staining, predominantly nuclear staining, or both cytoplasmic and nuclear staining are indicated by different arrows in Fig. 2I. In the superior cervical ganglion the nucleoli seemed distinct, but there was apparently more cytoplasmic and less nuclear staining than in the trigeminal ganglion. We have no explanation for this at present. One should note, however, that the two ganglia are anatomically and functionally different, one of them (cervical) being sympathetic and the other one being sensory. We conclude that the nectin-1 gene is widely expressed in sensory and autonomic neurons, both sympathetic and parasympathetic. The gene is possibly also active in some satellite cells.

Nectin-1 RNA in the spinal cord

Cross sections of the thoracic part of the spinal cord are shown in Fig. 3. Again, there was no hybridization with the sense probe (Fig. 3C). In contrast, the ventral and dorsal horns were distinct after hybridization with the antisense probe due to staining of numerous cells in the gray matter that were significantly larger than those cells reacting in the white matter (Fig. 3A). The largest cells, with several branches extending from their bodies, were present in the ventral horn and were assumed to be motor neurons (Fig. 3B). The smaller cells in the white matter (Fig. 3A) were similar to those presumed to be Schwann cells in the fibrous part of the peripheral ganglia (Fig. 2). The nectin-1 gene is thus active in the spinal cord.

Nectin-1 RNA in neurons of the central nervous system

Although Northern blots had indicated that nectin-1 RNA is present in the mouse brain (Menotti *et al.,* 2000; Satoh-Horikawa *et al.,* 2000; Shukla *et al.,* 2000) and the receptor protein was detected in the hypothalamus region by immunohistochemistry (Shukla *et al.,* 2000), the cells expressing the receptor gene were not unambiguously identified nor was their distribution in the various parts of the central nervous system investigated. Sagittal sections including brain stem, cerebellum, brain, and olfactory bulb were therefore prepared and examined by *in situ* hybridization. The results observed at low and high magnifications are presented in Figs. 4 and 5, respectively. Several structures known to contain high numbers of neurons were heavily stained after hybridization with the antisense probe and could easily be identified without counterstaining with hematoxylin–eosin. Anatomical areas consisting mainly of nerve fibers, however, were unstained or weakly stained. Hybridization with the antisense probe was detected in almost all parts of the section (A and C), but no signal was observed with the sense probe at either low (B and D) or high (results not shown) magnification.

Selected areas in Fig. 4 are shown at high magnifications in Fig. 5. Distinct and well-characterized anatomical structures were observed. In the olfactory bulb (Fig. A) glomeruli (g), lacking cell bodies but consisting of axons from olfactory receptors in synapses with tufted (t) and periglomerular (pg) cells, were unstained. Periglomerular cells as well as tufted cells in the external plexiform layer were stained after hybridization with the antisense probe. The deeper layers of cells expressing nectin-1 RNA corresponded to the mitral body layer (m) and the granule layer (gl), respectively. These various cell types in the olfactory bulb are all neurons.

The pattern of hybridization signals in the cerebral cortex (Fig. 5B) reflected the distribution of neurons. The outer unstained molecular layer seen in Fig. 4A consists of numerous nerve fibers and few cells. Underneath was a broad layer of heavily stained cells (labeled fc in Figs. 4A and 5B). The frontal cortex, shown at higher magnification in Fig. 5C, also exhibited this pattern of hybridization. Many of these cells were rather uniform in size, but scattered in between were cells that appeared smaller and more faintly stained. The apparent variation in size and staining could result from sectioning at different planes through cells with similar levels of hybridization, but would also be consistent with the fact that there are various types of neurons in the cortex.

Hippocampus (hi) and dentate gyrus (dg), consisting of large pyramidal and smaller granular neurons, respectively, contained large amounts of nectin-1 RNA (Fig. 5D). The difference in cell size in the two structures could easily be seen.

The three layers of neurons in the cerebellum are demonstrated in Fig. 5E. The outer molecular layer (ml), consisting mainly of axons and dendrites, contained a few stained cells. The large pyramidal cells (Purkinje cells, pc) underneath and the smaller granular cells (gc) in the central part showed strong hybridization signals.

Strong hybridization signals were also observed in several structures not described in detail, for example, in the thalamus and the hypothalamus, in the brain stem, and in many well-defined nuclear groups. The signals were always restricted to the neuronal cell bodies. Expression of nectin-1 RNA in nonneuronal cells was observed in the choroid plexus (Fig. 5F, cp), mostly in the superficial epithelial layer.

Evidence that the nectin-1 gene is mainly expressed in neurons was so far based upon size, shape, and anatomical distribution of the stained cells. Further evidence was obtained by identification of neurons and glial cells by their reactions with antibodies directed against marker molecules, specifically neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP). To investigate whether NSE and receptor RNA were localized to the same cell, consecutive sections were made of which one was subjected to *in situ* hybridization (Figs. 6A and 6B) and the adjacent one incubated with the anti-NSE

antibody (Figs. 6C and 6D). Note that counterstaining with hematoxylin–eosin was performed in Figs. 6C and 6D. NSE staining showed, probably for technical reasons, slight variation in intensity from one area to another of the section. This may explain the stronger staining in the left than in the right part of Fig. 6C. With this in mind, the pattern of cells containing receptor RNA and NSE, respectively, in the cerebral cortex (Figs. 6A and 6C) are strikingly similar. Astrocytes reacting with GFAP (data not shown) were mainly observed in layers superficial to, and deeper than, that containing strong hybridization signals. The majority of cells expressing nectin-1 RNA were consequently not astrocytes.

Portions of individual hippocampal cells present in adjacent sections (Figs. 6B and 6D) were identified by superimposing transparent copies of the pictures. Some of these cells are labeled with numbers and clearly contained both NSE (Fig. 6D) and nectin-1 RNA (Fig. 6B). One would not expect the staining pattern to be identical in the two sections. First, detectable reaction was not observed in all neurons. Second, the portions of a given cell present in two adjacent sections were not identical unless the cell was cut symmetrically, which is obviously not the case for a large proportion of the cells. Third, the expression of NSE and receptor RNA relative to one another might vary among neurons. The general patterns of NSE-containing cells and of cells expressing nectin-1 RNA were similar, however.

The results described thus indicated that the nectin-1 gene was expressed in neurons in all parts of the central nervous system. Additional but more limited expression in glial cells could not be excluded. Expression of nectin-1 RNA in the superficial epithelial layer of the choroid plexus was also noted.

DISCUSSION

Previous studies showed that nectin-1 RNA is present in human and mouse brain tissue and/or in cultured cells of neuronal origin (Geraghty *et al.,* 1998; Menotti *et al.,* 2000; Satoh-Horikawa *et al.,* 2000; Shukla *et al.,* 2000). The results presented here clearly demonstrate that neurons throughout the mouse peripheral and central nervous system express nectin-1 RNA *in situ*. Few other cell types in the nervous tissue examined gave hybridization signals with the nectin-1 probe as strong as those observed in neurons. The results suggested that Schwann cells in the peripheral nervous system and epithelial cells in the choroid plexus may also express nectin-1 RNA. We cannot rule out low levels of expression in other cell types nor is it possible to conclude that all neurons express nectin-1.

Most of the previous studies on nectin-1 expression have focused on epithelial cells (Miyahara *et al.,* 2000; Satoh-Horikawa *et al.,* 2000; Takahashi *et al.,* 1999). It has been shown that nectin-1, nectin-2, and nectin-3 localize to the sites of cadherin-based adherens junctions in epithelia, along with l-afadin, an actin-binding protein. Certain isoforms of nectin-1, nectin-2, and nectin-3 can bind to afadin through interactions of their cytoplasmic tails with a PDZ domain in l-afadin. Although nectin-1 protein was detected by immunohistochemistry in neuronal cells of the mouse central nervous system (Shukla *et al.,* 2000), it is not yet known how nectin-1 might be localized in neuronal cells. A recent report demonstrates that l-afadin can be detected in the mouse central nervous system localized to various sites, including a symmetrical concentration at the puncta adhaerentia-like junctions between the mossy fiber terminals and the dendritic trunks of pyramidal cells (Nishioka *et al.,* 2000). These results suggest that nectin-1 might also be found localized to these complex junctions and perhaps also to synaptic junctions.

The fact that nectin-1 RNA is expressed in many neurons suggests that nectin-1 could be the entry receptor used by HSV-1, HSV-2, and PRV to infect neurons and to spread through the nervous system via synapses. The other known entry receptors for these viruses fail to mediate infection by HSV-1 and HSV-2 (nectin-2 in the mouse), by PRV (HVEM), or by HSV-2 and PRV (3-*O*-sulfated heparin sulfate). Nectin-1 is the only known entry receptor that can efficiently mediate entry of all three viruses (Table 1).

The pathologic changes occurring in neurological disease caused by HSV or PRV in mice and HSV in humans can be quite extensive (even more extensive than would be predicted by the apparently focal symptoms often observed in humans) but rarely follows the broad distribution of nectin-1 RNA observed in this study. Of course, levels of nectin-1 RNA expression do not necessarily correlate directly with levels of protein expression and it remains to be determined whether nectin-1 is expressed at synapses or at other sites appropriate for viral entry and viral spread. Moreover, the mechanism by which alphaherpesviruses spread in the nervous system is poorly understood, although spread clearly follows synaptically linked pathways in many parts of the nervous system (reviewed by Enquist *et al.,* 1998). Transsynaptic spread requires that virions (or some form of genome-containing particle) exit from axonal termini and enter the synaptically linked neuron. Capsids and glycoproteins of HSV seem to be transported separately in the axons, suggesting final assembly of the virus particle in the terminus of the axon (Holland *et al.,* 1999; Miranda-Saksena *et al.,* 2000; Penfold *et al*., 1994).

The requirements for entry of virus into neuronal cells during invasion of (or after injection into) the nervous system and for transsynaptic spread may be different, at least for PRV. Both HSV-1 and PRV mutants deleted for gD must be transcomplemented with gD to infect most or all cell types, including neuronal cells. In the case of HSV-1, absence of gD expression prevented spread of

virus from retinal neurons in the rat (inoculated with transcomplemented virus into the vitreous body) to retinorecipient areas of the brain (Dingwell *et al*., 1995). On the other hand, absence of gD expression by a transcomplemented PRV mutant did not prevent spread of virus from motor hypoglossal neurons of mice via transsynaptic transfer to other parts of the nervous system (Babic *et al.,* 1993). Although additional studies must be done, including comparisons of HSV-1 and PRV mutants under similar experimental conditions, the possibility exists that HSV-1 depends upon gD and a gD receptor for transsynaptic spread, whereas PRV does not. It is probably relevant in this regard that entry of both HSV-1 and PRV into cells requires four glycoproteins (gB, gD, gH, and gL), whereas cell fusion requires the same four glycoproteins in the case of HSV-1 (Pertel *et al.,* 2001; Turner *et al.,* 1998) but only gB, gH, and gL in the case of PRV (Klupp *et al*., 2000). Thus, PRV may be able to use another viral ligand and receptor for virus-induced cell fusion as well as for transsynaptic spread and therefore not have to depend entirely on a gD receptor.

Other viral mutations, such as deletion of gE or gI, have no effect on viral entry but have been shown to restrict the spread of HSV-1 and PRV in the nervous system of mice (Dingwell *et al*., 1995; Enquist *et al.,* 1994; Husak *et al*., 2000). These glycoproteins function as heterodimers (gE-gI) and have a role in targeting progeny virions to appropriate regions of the cell for egress (Dingwell *et al.,* 1994; Johnson *et al.,* 2000). Thus, the restricted spread of gE/gI mutants in the nervous system is probably due to failure of virus to exit from the initially infected cells and therefore unavailability of progeny to spread to adjacent neurons. Much remains to be learned about the viral and host requirements for transsynaptic spread of the alphaherpesviruses. We predict that nectin-1 can be one of the relevant host receptors, at least for HSV.

MATERIALS AND METHODS

Antibodies and other reagents

Antidigoxigenin-AP, Fab fragments (catalog no. 1093274), and blocking reagent for nucleic acid hybridization (catalog no. 1096176) were purchased from Boehringer Mannheim (USA). Monoclonal mouse anti-human neuron-specific enolase (NSE) (code no. M 0873) and rabbit anti-cow glial fibrillary acidic protein (code no. Z 0334) were from DAKO (Denmark). The following Sigma (USA) products were used: diethyl pyrocarbonate (DEPC) (D-5758, EC no. 216-542-8), triethanolamine (T-1377, EEC no. 203-049-8), acetic anhydride (A-6404, EEC no. 203-564-8), dimethyl-dichlorosilane (EEC no. 200-901-0), and deionized formamide ACS reagent (F-4761). Proteinase K, PCR grade (85025028-09) was from Boehringer Mannheim, Germany. Nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (catalog no. 18280-06) were from Gibco BRL, USA.

Preparation of probes

Plasmid pCR13 containing the entire mouse nectin-1 (HveC) gene was kindly provided by Dr. Cynthia L. Rowe, Northwestern University. A 276-bp fragment from the ectodomain of the gene was amplified by PCR. The position of the fragment is shown in Fig. 1. Sequences for *Hin*dIII or *Xho*I were added at the 5′ and 3′ ends of the fragment, respectively. The 5′ primer (A) was 5–CTA-AGCTT-CGCTGGCCTGCATTG-3′and the 3′ primer (B) was 5′-GCTCGAG-TGTAGGTTCCTGCCA-3′. The amplified fragment was inserted into plasmid pcDNA3 (Invitrogen, USA) which was cut with *Hin*dIII and *Xho*I. Correct insert was confirmed by sequencing. Digoxigenin-labeled sense and antisense probes were made by transcription from the T7 and SP6 promoters, respectively, using the DIG RNA Labeling Kit (SP6/T7) from Boehringer Mannheim (catalog no. 1175025) as described by the manufacturer. To estimate the concentrations, aliquots of each probe and of various concentrations of standard RNA were crosslinked to a membrane which was subsequently incubated with an anti-DIG antibody and then with reagents for color development, as described later. The probes were stored at −80°C.

Animals and preparation of tissues

Six-week-old female BALB/c or adult NMRI mice were sacrificed under deep fentanyl-benzodiazepam anesthesia by cardiac perfusion with phosphate-buffered saline pH 7.2 (PBS) followed by perfusion with 4% paraformaldehyde in PBS. The spinal, superior cervical, pterygopalatine, and trigeminal ganglia, thoracic spinal cord, and brain were removed, fixed overnight in the same fixative, and then embedded in paraffin using an automatic processor. Six-micrometer sections were adhered to silanized glass slides (Oncor, USA) for *in situ* hybridization analysis. Some sagittal brain sections were bought from Novagen (Madison, USA; catalog no. 69143-3).

In situ hybridization

Coverslips were treated with dimethyl-dichlorosilane as described in the SureSite II System manual from Novagen. *In situ* hybridization was performed according to the procedure of Breitshopf and Suchanek (1996). Briefly, the paraffin was removed with xylene and the tissue rehydrated in a graded series of ethanol in water. The sections were then fixed again in 4% paraformaldehyde in PBS and rinsed with TBS (50 mM Tris–HCl (pH 7.5) and 150 mM NaCl) before incubation with 0.2 M HCl for 10 min at room temperature. After rinsing in TBS, acetylation was performed for 10 min at room temperature using 0.1 M triethanolamine (pH 8.0) and acetic anhydride at a final concentration of 0.25%. Sections were rinsed again in TBS and incubated with proteinase K. Optimal conditions for proteinase K treatment were

determined to be incubation for 20 min at room temperature at a concentration of 20 μg/ml. After rinsing in TBS the sections were dehydrated in a graded series of ethanol in water and stored at room temperature until further use. Hybridization was performed under coverslips in a humidified chamber for 4–6 h at 60°C using a hybridization solution containing 2× SSC, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.02% SDS, and 50% deionized formaldehyde. Forty-seven microliter hybridization solution and 1 μl probe was added per section. Posthybridization washes included incubation at 4°C overnight in 2× SSC followed by three 20 min washes at 55°C with 50% deionized formamide in 1× SSC and finally two 20-min washes at room temperature with 1× SSC before rinsing in TBS.

Incubation with antibodies, color development, and immunohistochemistry

The sections were incubated at room temperature for 15 min with 1% blocking reagent in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) followed by 60 min with anti-DIG antibody diluted 1:500 in blocking solution. Color was developed by soaking the section in an alkaline buffer (100 mM Tris–HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$) and then in a solution of NBT and BCIP as described by the manufacturer. Immunohistochemistry was performed in the automatic immunostainer DAKO TechMate 500 as described by the manufacturer using a DAKO ChemMate Detection Kit (peroxidase/DAB, code no. K 5001), which is based on an indirect streptavidin-biotin method. The primary antibodies were diluted 1:400 and incubation was for 25 min. Control reactions were performed with DAKO Rabbit Immunoglobulin fraction (code no. X0903) at a dilution of 1:950 as a substitute for GFAP and with DAKO Antibody Diluent (code no. S2022) as a substitute for NSE.

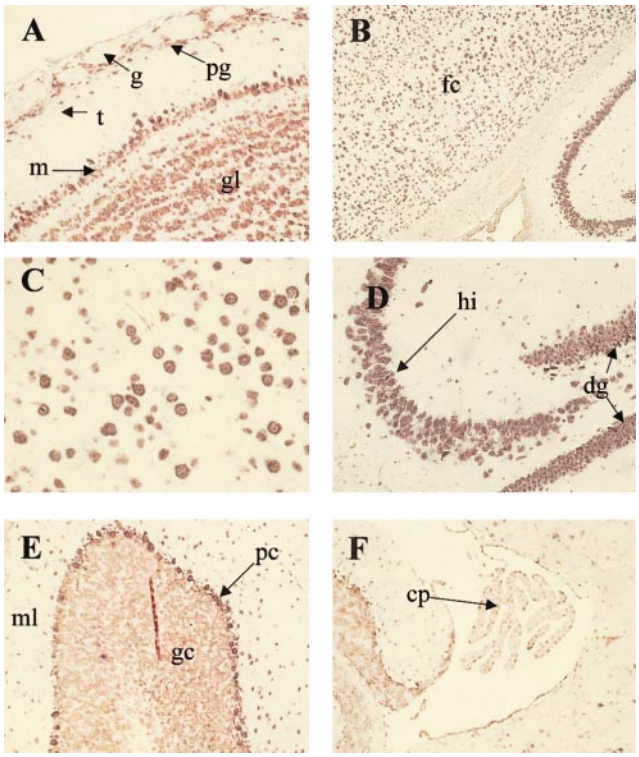

FIG. 5. *In situ* hybridization of different parts of the central nervous system as observed at higher magnifications. Regions hybridized with the antisense probe and indicated by the boxed areas in Fig. 4 (A and C) are shown at higher magnifications. (A) The olfactory bulb, magnification ×200; g, glomeruli; pg, periglomerular cells; t, tufted cells; m, mitral cells; gl, glomerular layer. (B) Cerebral cortex and hippocampus, magnification ×80; fc, frontal cortex. (C) Frontal cortex, magnification ×320. (D) Hippocampus (hi) and dentate gyrus (dg), magnification ×200. (E) Cerebellum, magnification ×200; ml, molecular layer; pc, Purkinje cells; gc, granular cells. (F) Choroid plexus (cp), magnification ×200.

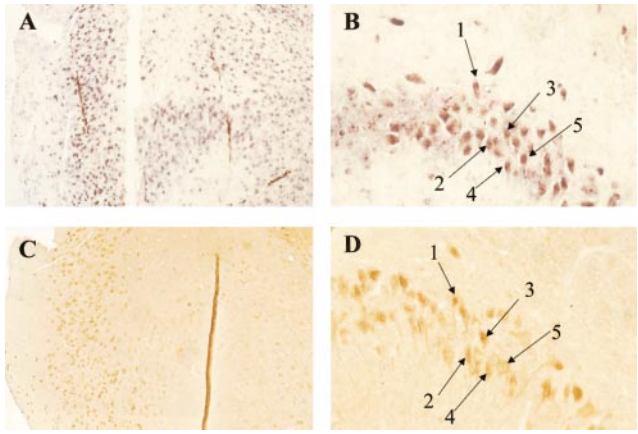

FIG. 6. Detection of nectin-1 mRNA in cells containing neuron-specific enolase. Consecutive sagittal sections of the brain were prepared. One of them was subjected to *in situ* hybridization (A and B) and the adjacent one incubated with an antibody against neuron-specific enolase (C and D). Additional staining with hematoxylin–eosin was performed in the sections used for enolase detection. Areas from the cerebral cortex are shown in A and C, and from the hippocampus in B and D. Magnifications were ×80 in the cortex and ×320 in the hippocampus area. Transparent images of the pictures in B and D were superimposed to identify cells which were cut so that portions of them were present in both sections. Some of these cells are labeled with numbers 1–5.

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